

Broad-Spectrum Peptide Inhibitors of Aminoglycoside Antibiotic Resistance Enzymes

David D. Boehr,¹ Kari-ann Draker,¹
Kalinka Koteva,¹ Manjeet Bains,²
Robert E. Hancock,² and Gerard D. Wright^{1,*}

¹Antimicrobial Research Centre
Department of Biochemistry
McMaster University
1200 Main Street West
Hamilton, Ontario L8N 3Z5
Canada

²Department of Microbiology and Immunology
University of British Columbia
6174 University Boulevard
Vancouver, British Columbia V6T 1Z3
Canada

Summary

The action of aminoglycoside antibiotics is inhibited by chemical modification catalyzed by aminoglycoside inactivating enzymes, which bind these cationic saccharides with active site pockets that contain a preponderance of negatively charged residues. In this study, it was observed that several cationic antimicrobial peptides, representing different structural classes, could serve as inhibitors of such aminoglycoside resistance enzymes. The bovine antimicrobial peptide indolicidin and synthetic analogs appeared to be especially effective against a range of resistance enzymes, inhibiting enzymes belonging to both aminoglycoside phosphotransferase and aminoglycoside acetyltransferase classes, where the mode of action was dependent on the class of antibiotic resistance enzyme. These peptides represent the first example of broad-spectrum inhibitors of aminoglycoside resistance enzymes.

Introduction

Clinical resistance to the aminocyclitol-aminoglycoside antibiotics is most often associated with the presence of inactivating enzymes. These enzymes, classified as aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs), or aminoglycoside phosphotransferases (APHs), catalyze the transfer of acetyl, adenosine monophosphate, or phosphate groups, respectively, onto the aminoglycoside antibiotics [1]. These modifications block the antibiotic's interaction with its target, the 30S ribosomal subunit [2], leading to a resistance phenotype. Aminoglycoside resistance is now widespread among clinically important bacteria and is impacting the utility of these bactericidal agents. Overcoming aminoglycoside resistance requires the development of new antibiotics that are impervious to modification by the enzymes, or in an alternative approach, the use of an aminoglycoside

alongside an inhibitor directed against the inactivating enzymes. Although antibiotics have been developed that resist modification by some inactivating enzymes, the large number and differing regiospecificities of group transfer presented by aminoglycoside modifying enzymes virtually guarantees that a new aminoglycoside will not be effective against all resistance mechanisms. Broad-spectrum inhibitors directed against more than one class of modifying enzyme would therefore be highly desirable and would allow the rescue of aminoglycoside antibiotic activity, analogous to the employment of β -lactamase inhibitors to overcome penicillin resistance [3].

The three-dimensional structures of representative members of all three classes of aminoglycoside modifying enzymes have directed our efforts to identify inhibitors active against resistance enzymes. For example, the aminoglycoside phosphotransferase APH(3')-IIIa was shown to be structurally homologous to eukaryotic protein kinases [4], prompting a screen of protein kinase inhibitors from which many compounds were determined to inhibit APHs [5]. The AACs and ANTs also have eukaryotic homologs, and in particular, AAC(3)-Ia [6] and AAC(6')-II [7] belong to the large GCN5-related N-acetyltransferase superfamily, which includes the eukaryotic histone acetyltransferases GCN5 and Hat1. These structural studies have correlated well with functional studies that have shown that APHs [8] and AACs [7] can phosphorylate and acetylate peptides, respectively. In general, positively charged peptides are favored, likely owing to the prevalence of negatively charged residues in the active site pockets of aminoglycoside modifying enzymes (Figure 1). The anionic depression present in the resistance enzymes is thought to attract positively charged antibiotics and act as an electronic scaffold for the efficient binding of a variety of aminoglycoside structures. Given this common binding site feature, positively charged molecules could be developed that interfere with this interaction with aminoglycosides and lead to broad-spectrum inhibition of resistance activity.

Given that aminoglycoside resistance enzymes share this common binding strategy and that many of them also bind peptides and proteins, we hypothesized that cationic peptides could serve as lead molecules in the development of a universal aminoglycoside resistance inhibitor. Cationic peptides are also good starting points for these studies, as many of them have antimicrobial activity themselves [9]. While the site of action for some of these peptides appears to be the bacterial cytoplasmic membrane, there is evidence for penetration of others into the cytoplasm where aminoglycoside resistance enzymes are active [10, 11].

In this study, we have screened a variety of cationic antimicrobial peptides against three well-characterized aminoglycoside resistance enzymes, APH(3')-IIIa, AAC(6')-II, and AAC(6')-APH(2''). APH(3')-IIIa (Kan^r) and AAC(6')-APH(2'') (Gent^r) are two of the most common and important resistance determinants in gram-positive bacterial pathogens [12], whereas AAC(6')-II is a chro-

*Correspondence: wrightge@mcmaster.ca

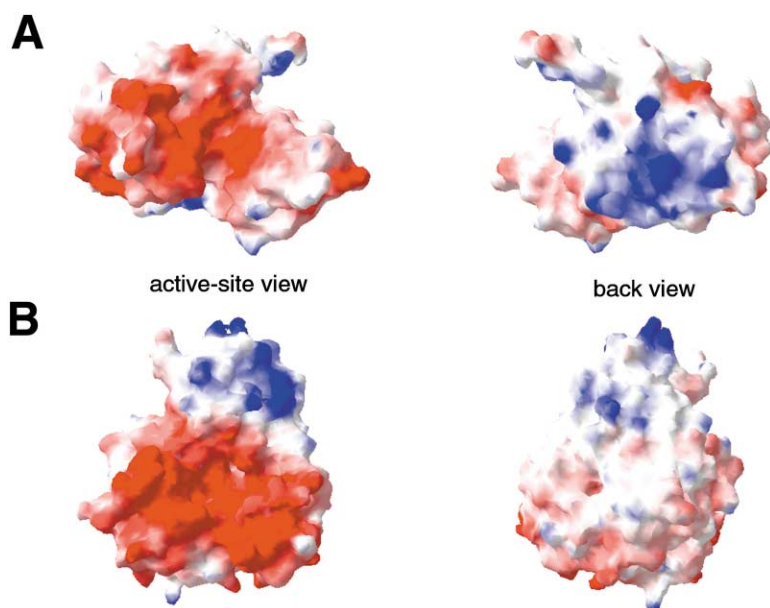


Figure 1. The Common Anionic Depression Found in the Active Sites of Aminoglycoside Resistance Enzymes

(A) Electrostatic surface potential map of the aminoglycoside acetyltransferase AAC(6')-II (PDB 1B87). Red and blue indicate the regions of the enzyme with the greatest electro-negative and electro-positive characters, respectively.

(B) Electrostatic surface potential map of the aminoglycoside phosphotransferase APH(3')-IIIa (PDB 1J71), with colors denoted as in (A).

mosomally encoded determinant found in the difficult-to-treat *Enterococcus faecium* that lends low-level intrinsic resistance to many aminoglycosides and obviates aminoglycoside/penicillin synergy in these organisms [13]. We have identified cationic peptide inhibitors of both aminoglycoside phosphotransferases and aminoglycoside acetyltransferases that show similar affinity for binding to the enzymes as natural substrates. Moreover, the bovine antimicrobial peptide indolicidin and its analogs were shown to inhibit enzymes belonging to both the aminoglycoside phosphotransferase and aminoglycoside acetyltransferase classes of resistance enzymes. These compounds represent the first broad-spectrum inhibitors of aminoglycoside modifying enzymes.

Results and Discussion

Antimicrobial Peptides Inhibit Aminoglycoside Resistance Enzymes

Several structural classes of cationic peptides were screened against three aminoglycoside modifying enzymes representing two classes of aminoglycoside resistance enzymes, the APHs and the AACs, which are most prevalent in gram-positive pathogens (Table 1). Most of these peptides, except protegrin 1 and gramicidin S, are predicted to be unstructured in free solution and only adopt the structures described in Table 1 upon interaction with membranes [14].

The two aminoglycoside acetyltransferases, AAC(6')-II and AAC(6')-Ie (the acetyltransferase activity associated with the bifunctional enzyme AAC(6')-APH(2'')), showed different peptide affinities (Table 1). Neither indolicidin nor its analogs, CP11CN or CP10A, appeared to inhibit AAC(6')-Ie, whereas these peptides were good inhibitors of AAC(6')-II. On the other hand, the α -helical-forming peptides CP29 and CP2600 effectively inhibited AAC(6')-Ie, but did not have an effect against AAC(6')-II. CP10A appeared to be the best inhibitor of AAC(6')-

II, whereas gramicidin S, a bacterium-derived cyclic decapeptide, was the best inhibitor of AAC(6')-Ie.

For the most part, the two aminoglycoside phosphotransferases surveyed, APH(3')-IIIa and APH(2'')-Ia (the kinase activity of the bifunctional AAC(6')-APH(2'') enzyme), had similar peptide inhibitor profiles (Table 1). The β -hairpin-structured peptide protegrin (PG1) appeared to be the best inhibitor of the aminoglycoside phosphotransferases, and indolicidin and its analog, CP10A, had efficient inhibitory activity against both enzymes. CP11CN weakly inhibited APH(3')-IIIa but did not show any activity against APH(2'')-Ia, suggesting a limited selectivity between the aminoglycoside phosphotransferases.

The best peptide inhibitors were not necessarily those with the greatest positive charge, demonstrating that inhibitory peptides make specific amino acid contacts with the enzymes and their interaction is not exclusively dictated by ionic interactions. Indolicidin and its analog, CP10A, were especially effective in inhibiting the resistance enzymes with IC_{50} s in the low to mid-micromolar range for three of the four enzyme activities (Table 1). The more highly charged indolicidin analog CP11CN was only effective against AAC(6')-II and APH(3')-IIIa, although it was a significantly weaker inhibitor of APH(3')-IIIa compared to CP10A and indolicidin.

An Aminoglycoside Acetyltransferase Can Modify Antimicrobial Peptides

AAC(6')-II has shown the ability to acetylate peptides [7], which correlates with its structural similarity to histone acetyltransferases. It was therefore important to determine if the aminoglycoside acetyltransferases could modify the peptides analyzed in our screen. This would be especially critical for understanding the mechanism of action for those peptides determined to be inhibitors in the initial screen.

Several Lys-rich cationic peptides were found to be

Table 1. Cationic Antimicrobial Peptides that Inhibit the Aminoglycoside Antibiotic Resistance Enzymes AAC(6')-II, AAC(6')-APH(2'), and APH(3')-IIIa

Peptide ^a	Sequence ^b	Charge at pH 7	Structure	References	IC ₅₀ (μM) ^c			
					AAC(6')-II ^c	AAC(6')-Ie	APH(2')-Ia	APH(3')-IIIa
CP29	KWKSFIKKLTAVKKVLTGLPALIS	+6	α-helical	[21]	n.a. ^d	21 ± 9	n.a.	n.a.
CP2600	KWKSFIKKLTSAKKVTTAAKPLTK	+7	α-helical	unpublished	n.a.	38 ± 9	n.a.	n.a.
CM3	KWKFKIKSLTKAAKTAVKTAKKPLIV	+9	α-helical	[21]	24 ± 1	n.a.	n.a.	n.a.
Indolicidin	ILPWKPWWPWRR-NH ₂	+4	Extended	[22]	13 ± 1	n.a.	64 ± 5	11 ± 2
CP11CN	ILKWPWWPWRRK-NH ₂	+6	Extended	[22]	23 ± 4	n.a.	n.a.	51 ± 10
CP10A	ILAWKWAWWARR-NH ₂	+4	α-helical	[22]	4.4 ± 0.2	n.a.	36 ± 6	11 ± 1
PG1	RGRLCYCRRRFCVCVGR	+6	β-hairpin	[23]	n.a.	n.a.	20 ± 4	5.2 ± 0.3
Gramicidin S	(cyclic) LF ^d PVOLF ^d PVO	+2	β-sheet	[22]	n.a.	14 ± 2	n.a.	n.a.

^a In addition to these peptides, the following α-helical peptides were screened against one or more aminoglycoside modifying enzymes (CP26, CEME, CEMA, CPα1, and CPα2) but did not show any significant inhibitory activity [22].

^b For gramicidin S, the superscript ^d indicates D enantiomer, and O is the one letter code for ornithine. All other amino acid residues are described by their one letter code.

^c For the aminoglycoside acetyltransferases, the IC₅₀s for AAC(6')-II and AAC(6')-Ie were determined in the presence of 50 μM acetyl CoA and 50 μM kanamycin A, and 100 μM acetyl CoA and 40 μM kanamycin A, respectively, using the aminoglycoside acetyltransferase assay described in Experimental Procedures. For the aminoglycoside phosphotransferases, the IC₅₀s were determined in the presence of 1000 μM ATP and 100 μM kanamycin A, using the kinase assay described in Experimental Procedures.

^d No inhibitory activity for this peptide against this enzyme target.

acetylated by AAC(6')-II (Table 2). The peptides displayed K_M values similar to those of aminoglycosides, but with much lower turnover rates, comparable to those of previously known peptide substrates, such as poly-L-lysine [7]. The observation that AAC(6')-II could catalyze acetylation of the antimicrobial peptides CM3 and CP11CN had no effect on their antibacterial properties when tested against bacteria expressing the AAC(6')-II resistance enzyme (data not shown). Thus, despite the fact that these peptides can be modified by AAC(6')-II, the presence of this aminoglycoside resistance enzyme does not confer resistance to these cationic peptides, auguring well for a "resistance-free" environment for the development of cationic antimicrobial peptides.

The peptides were also tested as substrates for AAC(6')-Ie, but no acetylation activity was detected (data not shown). This is consistent with our previous unpublished studies with AAC(6')-Ie, where we have been unable to identify a peptide substrate for this enzyme activity. AAC(6')-Ie and AAC(6')-II do not share a high sequence homology [15], and AAC(6')-Ie may be structurally different from AAC(6')-II in important ways, such that it is unable to accept peptide substrates. However, as evidenced by the inhibition data, both enzymes bound peptides and their aminoglycoside modifying activities were reduced by that interaction.

Indolicidin Analogs Inhibit Aminoglycoside Phosphotransferases and Aminoglycoside Acetyltransferases through Different Modes of Action

As indolicidin and its analog CP10A were able to inhibit both APHs and AAC(6')-II, we compared the mode of action between the two classes to gain insight into the structural requirements for efficient inhibition by these compounds. Toward the first goal, we determined the inhibition patterns with respect to AAC(6')-II and the two APH enzymes.

With AAC(6')-II, CP10A demonstrated a competitive inhibition pattern with respect to the aminoglycoside substrate kanamycin A (Figure 2A) and a noncompetitive pattern with respect to acetyl CoA (Figure 2B). This classical pattern of inhibition suggests that CP10A binds at or near the aminoglycoside binding pocket and does not make any interactions with the acetyl CoA binding pocket (Table 3).

In contrast, a more complex situation arises in inhibition patterns determined with the aminoglycoside phosphotransferases, APH(3')-IIIa and APH(2')-Ia, where the peptide inhibitors demonstrated noncompetitive patterns with respect to both aminoglycoside (Figure 2C) and ATP (Figure 2D, Table 3). These results suggest that the peptide inhibitors have multiple binding modes to the APH class of resistance enzyme. The inhibition pat-

Table 2. Acetylation of Cationic Peptides by AAC(6')-II

Substrate	K_M (μM)	k_{cat} (sec ⁻¹)	k_{cat}/K_M (M ⁻¹ sec ⁻¹)
Kanamycin A ^a	19.9 ± 8.8	0.816 ± 0.207	4.60 × 10 ⁴
Poly-L Lysine ^b	17.1 ± 1.4	0.004 ± 0.001	2.23 × 10 ²
CM3	27 ± 12	0.03 ± 0.01	1.1 × 10 ³
CP11CN	25 ± 14	0.04 ± 0.01	1.7 × 10 ³
MS178 ^c	16 ± 7	0.01 ± 0.00	2.7 × 10 ²

^a [19]

^b [7]

^c Peptide MS178 has the sequence of GIGKFLKAKKFGKATVKILKK (NH₂) in single amino acid letter code.

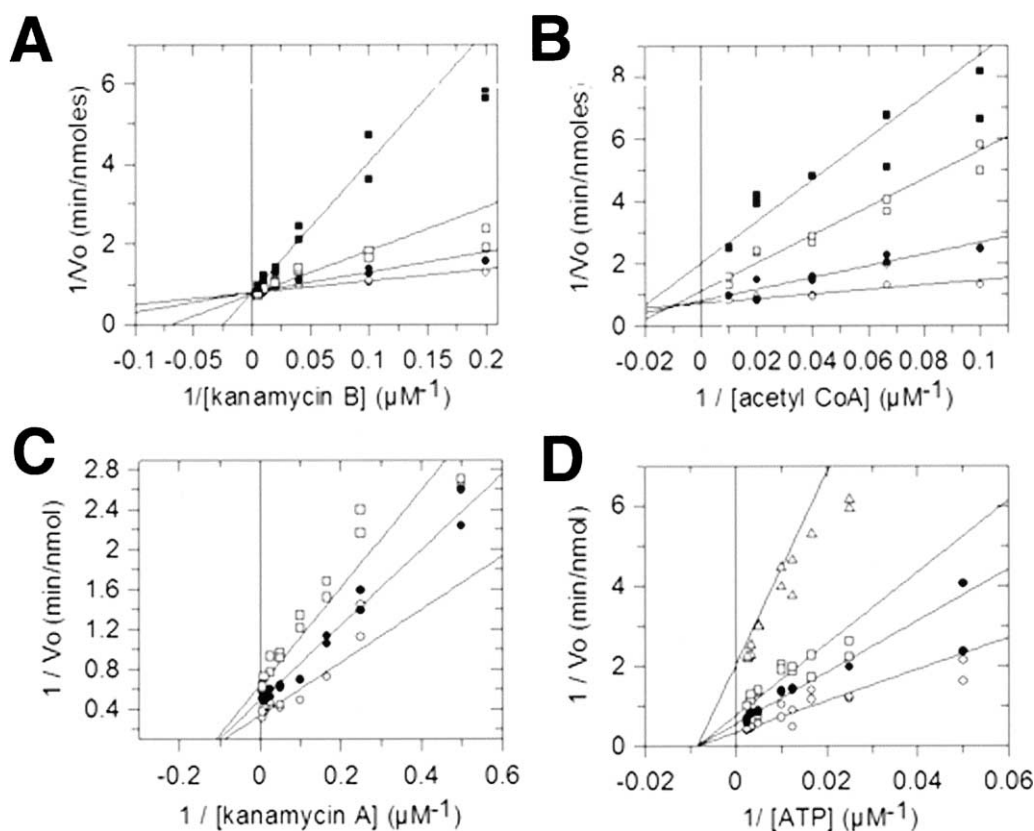


Figure 2. The Inhibition Patterns of Aminoglycoside Acetyltransferases and Phosphotransferases by the Peptide CP10A

(A) Inhibition of AAC(6')-II with kanamycin B as the variable substrate and acetyl CoA as the saturating substrate. The concentrations of CP10A are as follows: 0 (open circle), 5 (closed circle), 10 (open square), and 20 (closed square) μM .

(B) Inhibition of AAC(6')-II with acetyl CoA as the variable substrate and kanamycin B as the saturating substrate. The concentrations of CP10A are as follows: 0 (open circle), 10 (closed circle), 20 (open square), and 40 (closed square) μM .

(C) Inhibition of APH(2'')-Ia with kanamycin A as the variable substrate and ATP as the saturating substrate. The concentrations of CP10A are as follows: 0 (open circle), 5 (closed circle), and 10 (open square) μM .

(D) Inhibition of APH(2'')-Ia with ATP as the variable substrate and kanamycin A as the saturating substrate. The concentrations of CP10A are as follows: 0 (open circle), 5 (closed circle), 10 (open square), and 40 (open triangle) μM .

terns demonstrate that the peptides bind both to free enzyme and to enzyme-substrate complexes, and also suggests that the peptides do not bind simply to either the aminoglycoside binding site or the ATP binding pocket, but influence both sites directly or indirectly.

Structure-Activity Relationships of Indolicidin Analog CP10A

The indolicidin analog CP10A demonstrated similar affinity as aminoglycoside substrates, with K_s s in the mid to low micromolar range, to both AAC and APH classes of antibiotic resistance enzymes (Table 3). Our initial hypothesis was that these peptides would inhibit the aminoglycoside resistance enzymes by binding primarily through electrostatic interactions. Consistent with this hypothesis, increasing the salt concentration had a negative impact on IC_{50} for CP10A with both AAC(6')-II (Figure 3A) and APH(3')-IIIa (Figure 3B).

To gain further insight into the important interactions between the peptide and the enzymes, we assayed the resistance enzymes against a number of fragments of CP10A. A peptide consisting of the six N-terminal resi-

dues of CP10A (GW15) did not inhibit either APH(3')-IIIa or AAC(6')-II; however, a peptide composed of the five C-terminal residues (GW11) was able to weakly inhibit APH(3')-IIIa (Table 4). This suggests that the most important binding interaction to APH(3')-IIIa occurs with the C-terminal proportion of CP10A. However, inhibition was significantly increased as additional residues were added onto the N terminus, where the addition of hydrophobic residues had the largest impact on changes in IC_{50} (Table 4). This result suggests that hydrophobic interactions and van der Waals forces also play important roles in the interactions between peptide and resistance enzyme.

With either APH(3')-IIIa or AAC(6')-II, maximum inhibition occurred only with full-length CP10A (Table 4). CP10A is predicted to adopt an amphipathic helix structure with one face primarily hydrophobic and the other positively charged (Figure 3C). To determine the potential importance of this secondary structure and the placement of positively charged amino acid residues in the peptide, we tested the resistance enzymes against peptides GW27, which exchanges residues 5 and 7 in CP10A, and GW28, which exchanges residues 11 and

Table 3. <i>K_i</i> Determinations for Indolicidin and Its Analogs with the Aminoglycoside Resistance Enzymes					
Enzyme	Inhibitor	Variable Substrate	Pattern ^a	<i>K_{is}</i> (μM)	<i>K_{ii}</i> (μM)
APH(2'')-Ia	indolicidin	kanamycin A	NC	22.1 ± 1.7	^b
		ATP	NC	23.8 ± 3.4	^b
	CP10A	kanamycin A	NC	10.1 ± 0.8	^b
		ATP	NC	7.66 ± 0.61	^b
APH(3')-IIIa	indolicidin	kanamycin A	NC	14.6 ± 2.1	^b
		ATP	NC	10.3 ± 1.4	^b
	CP10A	kanamycin A	NC	30.5 ± 4.6	^b
		ATP	NC	13.8 ± 1.1	^b
	CP11CN	kanamycin A	NC	51.2 ± 5.2	^b
		ATP	NC	73.0 ± 28.2	^b
AAC(6')-II	indolicidin	ribostamycin	C	4.23 ± 0.96	
		acetyl CoA	NC	38.0 ± 1.7	^b
	CP10A	kanamycin B	C	2.41 ± 0.60	
		acetyl CoA	mixed	4.76 ± 1.33	31.7 ± 8.3

^a The patterns were either competitive (C), true noncompetitive (NC), or mixed inhibition and characterized according to Experimental Procedures.

^b In true noncompetitive inhibition, *K_{is}* = *K_{ii}*.

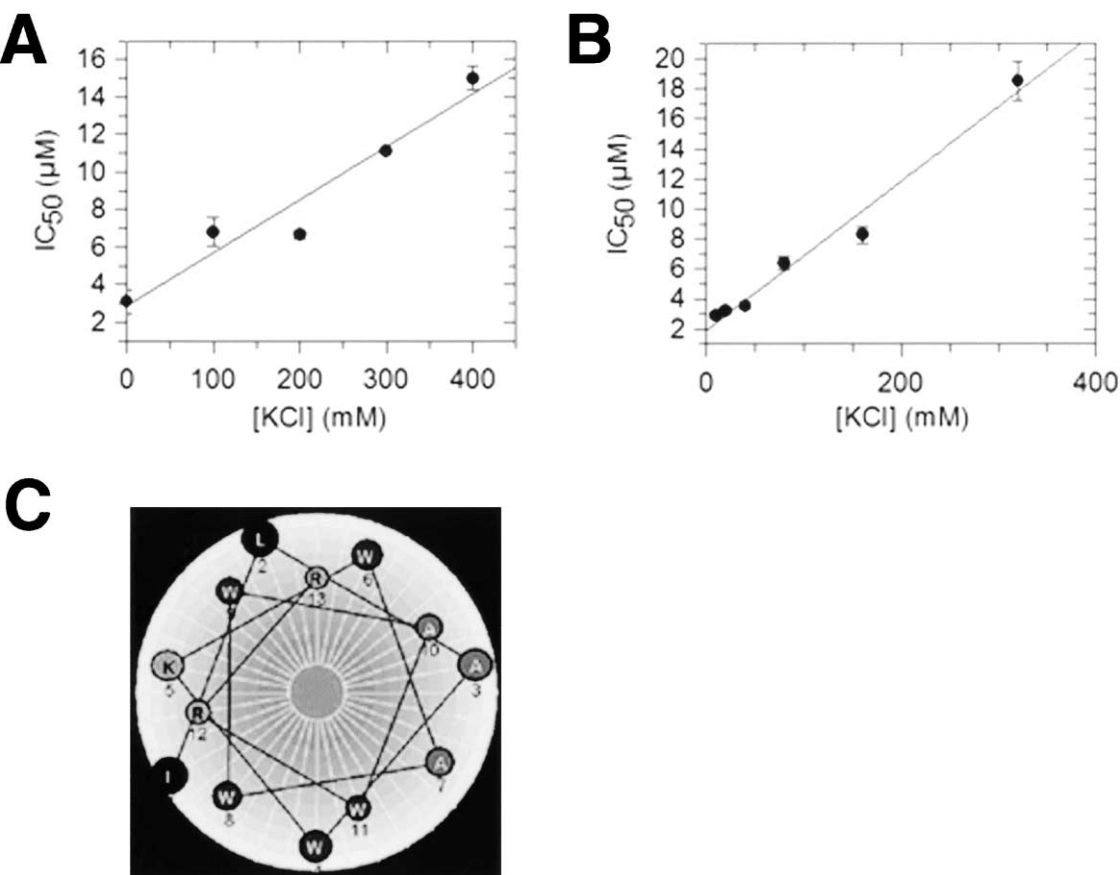


Figure 3. The Nature of the Interactions between the Peptide CP10A and the Aminoglycoside Resistance Enzymes AAC(6')-II and APH(3')-IIIa

(A) Effect of salt on the inhibitory activity of CP10A on AAC(6')-II. IC₅₀ were determined in the presence of 50 μM acetyl CoA and 50 μM kanamycin A.

(B) Effect of salt on the inhibitory activity of CP10A on APH(3')-IIIa. IC₅₀ were determined in the presence of 100 μM ATP and 60 μM kanamycin A.

(C) Helical wheel diagram for CP10A. CP10A has the ability to form an amphipathic helix with positive charged amino acid residues on one face and primarily hydrophobic amino acid residues on the other face. The propensity for α-helical formation increases in the presence of negatively charged lipids. This secondary structure may be important for the peptide's interactions with the negatively charged surface patches of aminoglycoside resistance enzymes.

Table 4. Structure-Activity Relationship for the Peptide CP10A with APH(3')-IIIa and AAC(6')-II

Peptide	Sequence	IC ₅₀ (μM) ^a	
		APH(3')-IIIa	AAC(6')-II
CP10A	ILAWKAWWAWRR	8.5 ± 0.7	4.4 ± 0.2
GW22	LAWKAWWAWRR	22 ± 1	10 ± 2
GW21	AWKAWWAWRR	16 ± 1	13 ± 1
GW20	WKAWWAWRR	31 ± 3	17 ± 3
GW19	KWAWWAWRR	79 ± 7	No inhibition
GW18	WAWWAWRR	55 ± 6	No inhibition
GW17	AWWAWRR	>100	No inhibition
GW16	WWAWRR	>100	No inhibition
GW11	WAWRR	~400	No inhibition
GW15	ILAWKA	No inhibition	No inhibition
GW27	ILAWAWKWAWRR	9.0 ± 1.4	10 ± 1
GW28	ILAWKAWWARWR	5.0 ± 0.5	5.8 ± 1.0

^aDetermined in the presence of 100 μM ATP and 60 μM kanamycin A for APH(3')-IIIa, and 50 μM acetyl CoA and 50 μM kanamycin A for AAC(6')-II.

12 in CP10A. These peptides have the same amino acid composition and charge as CP10A, but with disrupted amphipathic properties. GW28 was a slightly better inhibitor of APH(3')-IIIa than was CP10A; however, CP10A was still the best inhibitor of AAC(6')-II where GW27 was 2.3-fold less potent in inhibiting the enzyme than CP10A (Table 4). Considering these relatively minor effects on inhibition, the positioning of the positively charged amino acid residues has little impact on inhibitory activity against the resistance enzymes. This is not completely unexpected, as there are a number of negatively charged residues in the resistance enzymes that could interact with the positively charged amino acid residues in the peptides, depending on the nature of the peptide. This is encouraging as it suggests that there is enough flexibility in the peptide-resistance enzyme interaction such that the peptides have the ability to attenuate a broad range of resistance activity by adopting the most energetically favorable conformations dependent upon the available interactions in the different enzymes.

While there is evidence to suggest that some cationic peptides do enter the cell [10, 11], none of the peptides we examined in this study showed synergistic antimicrobial properties with aminoglycosides in organisms harboring resistance genes (not shown).

Significance

Aminoglycoside phosphotransferase and acetyltransferases inactivate their antibiotic substrates, rendering these important molecules clinically obsolete. One major complication of the treatment of aminoglycoside antibiotic resistance is the large variety of inactivating enzymes. However, aminoglycoside resistance enzymes share a common anionic depression in their active sites, and this common structural motif can be exploited in inhibitor design. We have demonstrated that positively charged peptides inhibit an array of aminoglycoside resistance enzymes. Moreover, indolicidin and its analogs potentially inhibit both phosphotransferases and acetyltransferases, although the mode of inhibition is different between the classes of enzyme. The peptides likely bind to the aminoglyco-

side binding pocket in AAC(6')-II, where the enzyme can acetylate selected peptides. This result suggests that if there are important intracellular targets for cationic antimicrobial peptides, their efficacy could be potentially diminished by promiscuous aminoglycoside acetyltransferases. For aminoglycoside phosphotransferases, the peptides appear to interact with both cofactor and aminoglycoside binding pockets, suggesting that they may bind in a variety of conformations, where both electrostatic and hydrophobic interactions play critical roles. These experiments set the stage for further structure-based development of the peptides to provide insight into the requirements of potent small molecule inhibitors capable of overcoming antibiotic resistance associated with a number of aminoglycoside modifying enzymes.

Experimental Procedures

Chemicals

β-NADH, phosphoenol pyruvate, and pyruvate kinase/lactate dehydrogenase enzymes were from Sigma (St. Louis, MO). Kanamycin A and gentamicin were from Bioshop (Burlington, ON, Canada). 4,4'-dithiodipyridine and acetyl CoA were from Amersham Pharmacia (Baie d'Urfe, PQ, Canada). Antimicrobial peptides were synthesized at the University of British Columbia's Nucleic Acid and Protein Sequencing Facility using N-(9-fluorenyl)methoxy carbonyl (Fmoc) chemistry and were purified by reversed phase high-pressure liquid chromatography. The peptide purity was confirmed by mass spectrometry.

APH(3')-IIIa [16], AAC(6')-APH(2') [17, 18], and AAC(6')-II [19] were purified as previously described.

APH and AAC Kinetic Assays

The phosphotransferase assay employed has previously been described [16]. The assay measures the production of ADP, generated upon aminoglycoside phosphorylation, and couples that production to the oxidation of β-NADH using the enzymes pyruvate kinase and lactate dehydrogenase. The rate of ADP production was determined by monitoring the decrease in absorbance at 340 nm.

Kanamycin acetylation by AAC(6')-II and AAC(6')-Ie was monitored by the in situ titration of free coenzyme A product with 4,4'-dithiodipyridine as previously described [17, 19]. Kinetic parameters for those cationic peptides behaving as AAC(6')-II substrates were determined using [1-¹⁴C] acetyl-CoA and a phosphocellulose binding assay described previously [7]. Reaction mixtures typically contained 25 mM HEPES (pH 7.5), 2 mM EDTA, 0.10 μCi [1-¹⁴C]Ac-CoA, and 40 pmoles pure AAC(6')-II with varying concentrations of poly-

L-lysine (as positive control) or cationic peptide and were allowed to proceed for 45 min.

Initial rates were fit to the Michaelis-Menton equation (1) using Grafit 4.0 [20]:

$$v = (k_{cat}/E_0)[S]/(K_m + [S]) \quad (1)$$

For the phosphotransferase assays, ATP was fixed at 1000 μ M and kanamycin A was fixed at 100 μ M when measuring the steady-state kinetic parameters for aminoglycoside substrate and ATP, respectively. For the acetyltransferase assays, acetyl CoA was held at 100 μ M when measuring the steady-state kinetic parameters for the cationic peptides.

IC₅₀ Determinations

IC₅₀ values for enzyme inhibition by various cationic peptides were determined for APH(3')-IIIa, APH(2'')-Ia, AAC(6')-Ie, and AAC(6')-II using the spectrophotometric kinetic assays described. Initial velocities in the presence of increasing concentrations of cationic peptides and appropriate concentrations of substrates were fit to the four parameter equation (2) using Grafit 4.0 software [20]:

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}}\right)^n} + Background \quad (2)$$

where *Range* is the fitted uninhibited rates minus background values, *y* is the enzyme rate, and *x* is the inhibitor concentration.

K_i Determinations for the Indolicidin Analogs

To determine K_s, steady-state kinetic parameters were determined in the presence of increasing amounts of peptide inhibitors and the data was fit to either a noncompetitive pattern (equation 3), a mixed pattern (equation 4), or a competitive pattern (equation 5) using Grafit 4.0 [20]:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (3)$$

$$v = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + \left(1 + \frac{[I]}{K_i}\right)[S]} \quad (4)$$

$$v = \frac{V_{max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (5)$$

Synthesis of Indolicidin-Based Peptides

All peptides were prepared by solid-phase peptide synthesis (0.5 mmol scale, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/hydroxybenzotriazole/diisopropylethylamine, alternated by dicyclohexylcarbodiimide/hydroxybenzotriazole activation) on Wang resin derivatized with appropriate C-terminal amino acid using 9-fluorenylmethoxy carbonyl-protected monomers. Side chain protecting groups used were *tert*-butoxycarbonyl for Lys and Trp, 2,2,4,6,7-pentamethylidihydro-benzofuran-5-sulfonyl for Arg. The peptides were cleaved from the resin using 0.1% H₂O in trifluoroacetic acid for 2 hr at room temperature. Reverse phase (C18) MPLC purification (20%–60% acetonitrile in 0.05% TFA/H₂O) over 2 hr afforded the peptides in more than 99% purity (by analytical HPLC) as a white solid. The identity of all peptide products were verified by electrospray mass spectrometry at the McMaster Regional Centre for Mass Spectrometry.

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References

- Wright, G.D., Berghuis, A.M., and Mobashery, S. (1998). Aminoglycoside antibiotics. Structures, functions, and resistance. *Adv. Exp. Med. Biol.* 456, 27–69.
- Llano-Sotelo, B., Azucena, E.F., Kotra, L.P., Mobashery, S., and Chow, C.S. (2002). Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. *Chem. Biol.* 9, 455–463.
- Livermore, D.M. (1995). β -lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.* 8, 557–584.
- Hon, W.C., McKay, G.A., Thompson, P.R., Sweet, R.M., Yang, D.S., Wright, G.D., and Berghuis, A.M. (1997). Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. *Cell* 89, 887–895.
- Daigle, D.M., McKay, G.A., and Wright, G.D. (1997). Inhibition of aminoglycoside antibiotic resistance enzymes by protein kinase inhibitors. *J. Biol. Chem.* 272, 24755–24758.
- Wolf, E., Vassilev, A., Makino, Y., Sali, A., Nakatani, Y., and Burley, S.K. (1998). Crystal structure of a GCN5-related N-acetyltransferase: *Serratia marcescens* aminoglycoside 3-N-acetyltransferase. *Cell* 94, 439–449.
- Wybenga-Groot, L.E., Draker, K., Wright, G.D., and Berghuis, A.M. (1999). Crystal structure of an aminoglycoside 6'-N-acetyltransferase: defining the GCN5-related N-acetyltransferase superfamily fold. *Struct. Fold. Des.* 7, 497–507.
- Daigle, D.M., McKay, G.A., Thompson, P.R., and Wright, G.D. (1999). Aminoglycoside antibiotic phosphotransferases are also serine protein kinases. *Chem. Biol.* 6, 11–18.
- Sitaram, N., and Nagaraj, R. (2002). The therapeutic potential of host-defense antimicrobial peptides. *Curr. Drug Targets* 3, 259–267.
- Matsuzaki, K., Yoneyama, S., and Miyajima, K. (1997). Pore formation and translocation of melittin. *Biophys. J.* 73, 831–838.
- Wu, M., Maier, E., Benz, R., and Hancock, R.E. (1999). Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* 38, 7235–7242.
- Miller, G.H., Sabatelli, F.J., Hare, R.S., Glupczynski, Y., Mackey, P., Shlaes, D., Shimizu, K., and Shaw, K.J. (1997). The most frequent aminoglycoside resistance mechanisms—changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups. *Clin. Infect. Dis.* 24 (Suppl 1), S46–62.
- Costa, Y., Galimand, M., Leclercq, R., Duval, J., and Courvalin, P. (1993). Characterization of the chromosomal *aac(6')-II* gene specific for *Enterococcus faecium*. *Antimicrob. Agents Chemother.* 37, 1896–1903.
- Friedrich, C.L., Rozek, A., Patrzykat, A., and Hancock, R.E. (2001). Structure and mechanism of action of an indolicidin peptide derivative with improved activity against gram-positive bacteria. *J. Biol. Chem.* 276, 24015–24022.
- Shaw, K.J., Rather, P.N., Hare, R.S., and Miller, G.H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* 57, 138–163.
- McKay, G.A., Thompson, P.R., and Wright, G.D. (1994). Broad spectrum aminoglycoside phosphotransferase type III from *Enterococcus*: overexpression, purification, and substrate specificity. *Biochemistry* 33, 6936–6944.
- Daigle, D.M., Hughes, D.W., and Wright, G.D. (1999). Prodigious substrate specificity of AAC(6')-APH(2''), an aminoglycoside antibiotic resistance determinant in enterococci and staphylococci. *Chem. Biol.* 6, 99–110.
- Boehr, D.D., Lane, W.S., and Wright, G.D. (2001). Active site labeling of the gentamicin resistance enzyme AAC(6')-APH(2'') by the lipid kinase inhibitor wortmannin. *Chem. Biol.* 8, 791–800.
- Wright, G.D., and Ladak, P. (1997). Overexpression and characterization of the chromosomal aminoglycoside 6'-N-acetyl-

- transferase from *Enterococcus faecium*. Antimicrob. Agents Chemother. 41, 956–960.
20. Leatherbarrow, R.J. (2000). Grafit 4.0 Ed. (Staines, UK: Erithacus Software).
21. Scott, M.G., Yan, H., and Hancock, R.E. (1999). Biological properties of structurally related alpha-helical cationic antimicrobial peptides. Infect. Immun. 67, 2005–2009.
22. Zhang, L., Rozek, A., and Hancock, R.E. (2001). Interaction of cationic antimicrobial peptides with model membranes. J. Biol. Chem. 276, 35714–35722.
23. Steinberg, D.A., Hurst, M.A., Fujii, C.A., Kung, A.H., Ho, J.F., Cheng, F.C., Louny, D.J., and Fiddes, J.C. (1997). Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. Antimicrob. Agents Chemother. 41, 1738–1742.